The enhancement of radiosensitivity in human esophageal squamous cell carcinoma cells by zoledronic acid and its potential mechanism

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Abstract Esophageal squamous cell carcinoma (ESCC) has a low 5-year patient survival rate. Radiotherapy, as a preoperative or postoperative treatment of surgery, has a crucial role in improving local control and survival of ESCC. Various chemotherapeutic and biologic agents have been used as radio-sensitizers in combination with radiotherapy. Here, we demonstrate that zoledronic acid (ZOL) has a radio-sensitizing effect on ESCC cells. Exposure of ESCC cancer cells to ZOL plus radiation resulted in increased cell death through arresting the cell cycle between S and G2/M phases. ZOL appeared to inhibit proliferation, tube formation and invasion of endothelial cells. These anti-angiogenic effects were more marked concurrently with irradiation. In addition, synergistic suppressive effects on VEGF

expression were observed after combined treatment. Our data suggest that the combination of ZOL and radiation is a promising therapeutic strategy to enhance radiation therapy for ESCC patients.

Keywords ESCC · Bisphosphonate · Radiosensitivity · Combined effect

Introduction

Esophageal carcinoma is a malignancy with remarkable geographic and racial distributions worldwide (Kamangar et al. 2006). In spite of the increasing

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morbidity of esophageal adenocarcinoma (EAC) in western countries, esophageal squamous cell carcinoma (ESCC), which possesses distinct pathologic characteristics from EAC, is the predominant histological type globally in many developing countries, particularly in Asia (Holmes and Vaughan 2007). Currently, surgery is the first choice for ESCC treatment, but patients are usually not diagnosed until their tumors have reached advanced stages, when radical esophagectomy is unfeasible (Jemal et al. 2005). Almost three fourths of the whole ESCC patients would be treated by radiotherapy due to their advanced stage of tumors. However, due to the widely differing susceptibility to radiotherapy exhibited in ESCC patients, there has been no significant improvement in the survival rates of patients treated with radiotherapy. Moreover, increasing the dose of radiation could not promote the local control rate of ESCC (Minsky et al. 2002). Therefore, identification of reliable radio-sensitizers to ESCC cells would be urgently desirable and has long been sought.

Bisphosphonates (BPs) are effective inhibitors of bone resorption and have been used for the past three decades in the treatment of osteoclast-mediated bone diseases (Jantunen 2002). The high concentrations of BPs that accumulate in bone being taken up by osteoclasts, specifically inhibit ATP-dependent enzyme systems, induce apoptosis by inhibiting the mevalonic acid pathway, and thus disrupt the biochemical processes that lead to bone destruction (Dunford et al. 2001). Recently, the anti-tumor effects of BPs have been widely reported, especially in combination therapy with various anti-tumor drugs. For instance, zoledronic acid (ZOL), one of the third-generation BPs, exhibits its synergistic functions in combination with chemotherapy in various cancer cell lines in vitro and in vivo (Matsumoto et al. 2005; Coleman et al. 2010; Zhao et al. 2012; Koto et al. 2010). Results revealed that ZOL halted the cell cycle at the S-phase or G₂/M transition, subsequently resulting in cell apoptosis. This ability of ZOL raises the possibility of ZOL as a potential radiosensitizer because cells in G₂/M are more sensitive than cells within other cell cycle phases.

Combined chemotherapy and radiotherapy for cancer treatment has already been used clinically. Likewise, combined therapy using ZOL and radiotherapy may further inhibit cell growth, and decrease the drug concentration and amount of irradiation.

Thus, combination of ZOL and radiotherapy would ultimately be safer than using ZOL or radiotherapy alone, with lower side effects (Ryu et al. 2010). Increasing evidence revealed ZOL serving as a radiosensitizer for the therapy of multiple cancer cell lines, for example, breast cancer, prostate cancer, myeloma and lung cancer (Ural et al. 2006; Algur et al. 2005; Ural and Avcu 2005). Nevertheless, to our knowledge, the mechanism of radiosensitization by ZOL in ESCC cells has not been reported. In the present study, we evaluated the potential of ZOL as a radiosensitizing reagent on ESCC cells. Herein are also reported in vitro data, for the first time, on the activity of ZOL plus radiation to endothelial cells.

Materials and methods

Cell lines and reagents

Two established human ESCC cell lines (EC109 and EC9706) and one endothelial cell line (CRL-1730) were maintained in our laboratory. These cell lines were routinely cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) supplemented with 10 % heat-inactivated fetal bovine serum (Gibco), 10 mmol/L glutamine, 100 U/mL penicillin (Sigma, St. Louis, MO, USA), and 100 µg/mL streptomycin (Sigma) in a humidified 5 % CO₂ atmosphere containing at 37 °C. One human immortalized normal esophageal epithelial cell line (Het-1A), which was used as a control for ESCC cell lines, was cultured in Defined Keratinocyte-SFM medium with growth supplements (Gibco). ZOL used in this study was supplied as the hydrated disodium salt by Novartis Pharma AG (Basel, Switzerland) and dissolved in phosphate-buffered saline (PBS) and used at a final concentration in a range of 0–64 mM. Stock ZOL solutions were aliquoted and kept at –20 °C for long-term storage. All dose formulations were prepared on the day of usage.

Cell proliferation assay

Cell proliferation was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, as described previously (Chen et al. 2010). Briefly, cells were cultured in 96-well plates (Costar, Cambridge, MA, USA) at a

density of 4×10^3 cells per well in triplicate. One day later, the cells were treated with ZOL at increasing concentrations. After additional 72 h incubation, 20 μ L of 5 mg/ml MTT (Sigma) was added 4 h prior to the time points when the supernatant was replaced by 150 μ L of DMSO for each well. The colored formazan product was measured at 490 nm using a micro-plate reader.

Colony formation assay

The analysis of clonogenicity was conducted to assess the percentage of surviving cellular clones after exposure to ZOL $\pm$ ionizing radiation (IR). Cells were plated at different densities in 6-well plates and permitted to attach for 24 h. Then the cells were treated with ZOL at the concentration of 8 μ M for 6 h and transiently exposed to graded doses of irradiation (from 0 to 6 Gy, 1 Gy per minute) using the Gamma-cell 3,000 Elan system (MDS Nordion Inc., Ontario, Canada). Then the medium was changed 24 h after radiation exposure and the colony formation assay was performed (incubated for 10–14 days). Thereafter, cells were stained with 0.5 % crystal violet in absolute ethanol, and colonies that contained more than 50 cells were counted. Each surviving fraction (SF) was calculated by dividing the number of cellular clones by the number of cells plated per plates, then by normalizing the SF for each radiation dose by the SF of non-treated cells.

Cell cycle analysis

Briefly, cells were treated with ZOL (8 μ M) 24 h after plating, then exposed to single IR dose (4 Gy) on the next day (24 h later), then fixed in 70 % ethanol and incubated overnight at -20°C . Cells were then washed and resuspended in 500 μ L of staining solution (50 μ g/mL of propidium iodide (PI), 100 μ g/mL RNAase and 0.2 % Triton X-100) for 30 min. The fluorescence associated with PI-bound DNA was measured by flow cytometry (Beckman Coulter, Cytomics FC 500, Brea, CA, USA). The cell cycle profiles were calculated using Multi-Cycle software.

Endothelial tube assay

This procedure has been previously described (You et al. 2008). Briefly, matrigel (Collaborative Biomedical,

Bedford, MA, USA) was added (100 μ L) to each well of a 96-well plate and allowed to be polymerized. Endothelial cells were exposed to ZOL (4 μ M), IR (4 Gy), or ZOL + IR for 24 h, and then seeded at a density of 5,000 cells per well into a plate 24 h after radiation treatment. The number of tubes per well was counted by two independent investigators using a CK2 Olympus microscope and averaged.

Cell invasion assay

The invasive capacity of endothelial cells after treatment with ZOL or/and IR was determined by an invasion chamber assay. Briefly, after treatment with ZOL (4 μ M), IR (4 Gy), or ZOL + IR for 24 h, 3×10^4 cells were re-suspended in 200 μ L serum-free DMEM and seeded into the insert chamber of a 24-well-size Matrigel-coated micropore membrane filter with 8 μ m pores (Millipore, Billerica, MA, USA), and the bottom chamber was filled with 750 μ L DMEM medium containing 10 % fetal bovine serum. After 48 h of incubation at 37°C , the inserts were removed and the non-invading cancer cells that remained on the upper side of the filter were scraped off. The membranes were fixed and stained with crystal violet reagent (Sigma) before counting in five $100 \times$ fields under phase-contrast microscopy.

Action of ZOL on VEGF expression

Cells were harvested after the indicated treatments and analyzed for VEGF-A expression by western blot and enzyme-linked immunosorbent assay (ELISA), as described previously (You et al. 2008; Ran et al. 2011). For western blot, 50 μ g of prepared protein for each sample was separated on a 12 % polyacrylamide gel, transferred onto a PVDF membrane (Millipore) and then subjected to western blot using the mouse monoclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were detected using the enhanced ECL kit (Amersham, Arlington Heights, IL, USA). For ELISA to detect the levels of secreted VEGF protein in culture supernatants, cells were rinsed with PBS and incubated in serum-free medium for 48 h. The culture supernatants were then collected and assayed using Quantikine kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistical analyses

All statistical analyses were performed using the statistical software package SPSS 17.0. Statistical significance was determined using a Student's *t* test and *P* values less than 0.05 were considered significant. Each experiment was performed in triplicate.

Results

ZOL inhibits the proliferation of both tumor cells and endothelial cells

The anti-proliferative effect of ZOL on ESCC cells was examined in the present study. Cell viability assays demonstrated that the activity of ZOL depended on the cell lines. After 72 h treatment, EC109 cells were more sensitive to a range of concentrations of ZOL, whereas EC9706 cells were relatively unaffected even exposed to high concentration of the drug. This result suggests that EC9706 cells were marginally more resistant compared to EC109 cells. However, normal esophageal epithelial Het-1A cells were not sensitive to this drug. We also found that

ZOL treatment was toxic to endothelial CRL-1730 cells, in a dose-dependent manner (Fig. 1).

ZOL enhances radiation-induced clonogenic suppression

Colony formation assays were used to further confirm whether ZOL pretreatment could enhance the sensitivity of ESCC cells to IR. The concentration of 8 μM for ZOL pre-treatment was chosen, because this low dose of ZOL potentially allowed evidencing a radio-sensitizing effect without exposing cells to excessive toxicity, according to the previous results. As shown in Fig. 2, IR was readily cytotoxic to both EC109 and EC9706 cells. The combination of lower dose of ZOL and IR resulted in significantly higher anti-tumor effects compared to treatment with IR alone. Nevertheless, this effect was abolished in Het-1A cells.

S phase arrest induced by ZOL treatment combined with IR

To determine the mechanism by which ZOL + IR induced combined inhibitory effect on ESCC cells, we analyzed the cell cycle progression by flow cytometry.

Fig. 1 ZOL affects cell proliferation capacity of ESCC and endothelial cells. Cells were treated with ZOL at increasing doses and proliferation capacity was measured using MTT assays. Data are expressed as mean $\pm$ standard deviation (SD). The results were representative of three independent experiments. **P* < 0.05, compared to the control (without addition of ZOL)

Fig. 2 ZOL affects clonogenic survival synergistically with IR in ESCC cells. Cells were treated with ZOL (8 μ M) for 6 h and then exposed to IR (4 Gy). Media were changed the day after and cells were maintained incubated for 10–14 days before counting. Each surviving fraction (SF) was calculated by

dividing the number of cellular clones by the number of cells plated per plates, then by normalizing the SF for each radiation dose by the SF of non-treated cells. Clonogenic curves demonstrate synergic activity between ZOL and ionizing radiation

Fig. 3 Effects of ZOL + IR on cell cycle distribution. EC109 and EC9706 cells were treated with ZOL (8 μ M), IR (4 Gy) or ZOL + IR, then harvested and stained by PI and analyzed by flow cytometry assay, which was performed 24 h after IR. The cell cycle profiles were determined with Multi-Cycle software. Difference between either ZOL or IR alone and combination of ZOL + IR was significant (* $P < 0.05$ for both)

The results revealed an increase of cell number in the S phase after ZOL treatment as compared with control, in both EC109 and EC9706 cell lines. Moreover, this effect was augmented significantly when combined treatment with ZOL + IR was performed to cells (Fig. 3). Additionally, a slight increase in subG1 phase was also observed in both cell lines as regard to

concurrent exposure to ZOL + IR, implicating that early cell death may be another major mechanism of the drug toxicity.

ZOL inhibits neovessels formation of endothelial cells

When endothelial cells are cultured on Matrigel matrix, they rapidly align and form hollow tube-like structures. To investigate whether the combination of ZOL and IR would exert synergism on neoangiogenesis, we analyzed the endothelial cells by tube formation assay. ZOL affected the tube formation ability of CRL1730 cells when they were given ZOL 24 h after plating. It was also found that the framework of the remaining neovessels was thinner after drug exposure. As expected, the combined treatment with ZOL + IR inhibited endothelial tube formation more significantly as compared with ZOL or IR at individual use (Fig. 4). This result suggests that combining ZOL with IR would yield an additive toxicity towards the neoangiogenic process.

ZOL suppresses invasiveness of endothelial cells

Subsequently, invasion assay was conducted to further confirm the synergistic inhibitory effect of ZOL and radiation on invasiveness of endothelial cells. The results revealed that the invasive capacity was significantly suppressed with ZOL treatment, as compared with the control. Although the inhibitory effect was not so obvious by IR alone, the combined treatment of IR plus ZOL showed a more marked inhibition on cell

Fig. 4 ZOL inhibits endothelial tube formation. CRL-1730 cells were treated with ZOL (4 μ M), IR (4 Gy), or ZOL + IR and then allowed to form tubes on Matrigel-coated plates. The number of tube branches (three independent wells) in low power field was counted and averaged. Difference between either ZOL or IR alone and combination of ZOL + IR was significant ($\star P < 0.05$ for both)

invasiveness when compared to ZOL or IR alone (Fig. 5). This association was synergistic, which means that the combination of the drug and IR produced an effect greater than the sum of their individual effects.

ZOL suppresses VEGF expression in combination with IR

It has been reported that ZOL exhibited indirect anti-angiogenic effects by suppressing the vascular endothelial growth factor (VEGF) expression in cancer cells, in a dose-dependent manner (Di Salvatore et al. 2011).

Fig. 5 ZOL inhibits invasion of endothelial cell. CRL-1730 cells were treated with ZOL (4 μ M), IR (4 Gy), or ZOL + IR and then seeded into the bottom chamber of Matrigel-coated micropore membrane filters. Penetrating cells were fixed, stained with crystal violet and counted under the microscope in five $100\times$ fields. Quantified data are expressed as mean $\pm$ SD. Difference between either ZOL or IR alone and combination of ZOL + IR was significant ($\star P < 0.05$ for both)

Therefore, VEGF expression and secretion were evaluated in EC109 cells subjected to combined treatment of ZOL + IR, by Western blot and ELISA assay, respectively. As shown in Fig. 6, no significant difference in VEGF expression was observed in cells treated with ZOL or IR alone, when compared to the control. However, combining ZOL with IR resulted in a more significant decrease in VEGF expression and secretion, compared to the individual use.

Fig. 6 Suppression of VEGF expression by combined treatment of ZOL + IR. EC109 cells were treated with ZOL (8 μ M), IR (4 Gy), or ZOL + IR and then the expression of secreted VEGF protein was detected by Western blot (*left*) and ELISA

(*right*) analyses. Quantified data are expressed as mean $\pm$ SD. Difference between either ZOL or IR alone and combination of ZOL + IR was significant (* P < 0.05 or both)

Discussion

It is well established that among BPs, ZOL has the strongest activity of anti-bone resorption and shows diverse direct anti-cancer effects (Senaratne et al. 2000; Yuasa et al. 2007). Owing to a high affinity for mineralized bone, ZOL rapidly localizes to bone, resulting in therapeutically effective local concentrations for the cancer cells in bone. ZOL inhibits farnesyl pyrophosphate synthase, consequently suppresses prenylation of small G-proteins such as Ras, Rho and Rab, reduces the signals they mediate, and thereby prevents malignant behaviors of cancer cells (Caraglia et al. 2007). The clinical use of the potent ZOL has increased recently (Rosen et al. 2003; Hirsh et al. 2008; Eidtmann et al. 2010). However, a key obstacle in clinical use is that the IC₅₀ is high because of its rapid clearance from the circulation within a few hours (Pandya et al. 2010). Current usage of ZOL trends in cancer therapy is in combination with anticancer agents including chemotherapeutic drugs, molecular targeted agents and other biological agents, resulting in synergistic responses as well as the ability to use lower and less toxic concentrations of these drugs.

On the other hand, increasing evidence revealed the combined effects of the third generation of BPs with standard radiotherapy as a potent sensitizer in various cancer cell lines. However, to our knowledge, it is not known whether similar synergistic effects exist with radiation in ESCC cells. In this study, we focused on the combined effect of ZOL and radiation, and

demonstrated that ZOL and IR were synergistic in inducing growth inhibition in ESCC cells. We used a relatively lower concentration of ZOL because our purpose was to radiosensitize cells, not to have direct antitumor activity. As expected, the lower dose of ZOL for combined use in this report could augment the effect of radiotherapy as well as decrease the side effects and complications, consistent with the previous report on prostate and myeloma cell lines (Algur et al. 2005). This is the first report to show that ZOL sensitizes ESCC cells to radiotherapy, indicating its clinical potential as a promising radiosensitizing agent for ESCC therapy.

Cancer cells are usually most sensitive during late S phase and G₂/M phase for radiotherapy (Sinclair 1968). A prominent G₂/M arrest occurs when cancer cells are treated with IR alone. In parallel, the S-phase might be decreased (Feng et al. 2010). It is reported that multiple myeloma cells showed an increase in the proportion of cells in the S-phase after exposure to ZOL, possibly due to slowing of progression through S phase or a block between S and G₂/M in cell cycle (Matsumoto et al. 2005). In this regard, ZOL leads to cell arrest in the G₂/M phase or to prolong cell cycle progression, implicating the possibility as a potential cell cycle radio-sensitizer (Ural et al. 2003). Thus, we further evaluated the mechanism of the combined effects of ZOL and radiation against ESCC cells. The combined treatment with a lower dose of ZOL plus radiation induced an increase in S-phase cells as well as in sub-G₁ early apoptosis cells, although the increased percentage of cells in sub-G₁ cells is not

sufficient to exert significant impact on the whole cell cycle. This result is consistent with a previous report on fibrosarcoma cells (Koto et al. 2010). Because ZOL demonstrates significant cytotoxic effects to cancer cells, it is reasonable to consider that multiple mechanisms, not limited to cell cycle, are involved in its anticancer process.

Angiogenesis is one of the critical components in cancer initiation and progression (Folkman 1995). Although these in vitro effects should be validated through in vivo assays, the inhibition of angiogenesis pathway may contribute at least partially to the augment of IR-induced growth delay after ZOL treatment, based on the fact that ZOL is also a potent inhibitor of angiogenesis through modification of various properties of endothelial cells for angiogenesis (Basi et al. 2010; Yamada et al. 2009). However, few previous reports have demonstrated the feasibility of combined treatment of ZOL and IR to endothelial cells. To test this possibility, we used established in vitro angiogenesis models, the vessels formation assay and invasion assay, to elucidate the fact that ZOL could exert its radio-sensitizing properties to inhibit angiogenesis. The direct inhibitory effect of ZOL on normal vasculature, especially in combination with radiation, could potentially overcome the intrinsic resistance of some tumor cells to the drug.

Further, increasing evidence has indicated that VEGF is the most potent and specific angiogenic factor and is closely associated with the growth, metastasis, and prognosis of cancers. It is also suggested that the expression of VEGF in tumors is up-regulated after radiotherapy, which is thought to be a defense mechanism against radiation (Hovinga et al. 2005). Consistently, cancers with overexpressed VEGF are insensitive to radiotherapy. Therefore, there was an urgent need for approaches that enhance the radiosensitivity of cancer cells by suppressing the expression of VEGF. In this study, we observed that combined treatment of ZOL + IR significantly exhibited its indirect anti-angiogenetic effects by suppressing VEGF expression and secretion derived by cancer cells, which might be another important mechanism underlying the enhanced radiosensitivity by ZOL.

In conclusion, the combination of ZOL and radiation leads to enhanced anti-tumor activity in human ESCC cells through halting the cell cycle in S phase. The combined therapy also demonstrated the significant anti-angiogenetic effects through suppressing

endothelial cells and down-regulating VEGF expression by cancer cells. To our knowledge, this is the first report for investigating the combined effect of ZOL and radiation in ESCC cells and endothelial cells. Our findings suggest that combined anti-cancer treatment of ZOL with IR would be a promising therapeutic option for patients with ESCC, although further in vivo and clinical studies are necessary to confirm these results in the future.

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Conflict of interest None declared.

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